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Beta-2-glycoprotein-1 and alpha-1-antitrypsin as urinary markers of renal cancer in Von Hippel-Lindau patients

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Running head: Markers of renal cancer in VHL syndrome patients

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Abstract

CONTEXT Von Hippel-Lindau disease (VHLD) is a rare inherited neoplastic syndrome. Among all the VHLD-associated tumors, clear cell renal cell carcinoma (ccRCC) is the major cause of death. OBJECTIVE The aim of this paper is the discovery of new non-invasive biomarker for the monitoring of VHLD patients. MATERIALS AND METHODS We compared the urinary proteome of VHLD patients, ccRCC patients and healthy volunteers. RESULTS Among all differentially expressed proteins, A1AT (alpha-1-antitrypsin) and APOH (beta-2-glycoprotein-1) are strongly over-abundant only in the urine of VHLD patients with a history of ccRCC. DISCUSSION AND CONCLUSION A1AT and APOH could be promising non-invasive biomarkers.

Keywords

Von-Hippel Lindau disease, renal cancer, beta-2-glycoprotein-1 and alpha-1-antitrypsin, urine, proteomics

Introduction

The Von Hippel-Lindau (VHL) disease is a rare autosomal dominant inherited neoplastic syndrome (López 2013). The gene responsible for the VHL disease (VHLD) is a tumor suppressor gene encoding for a multifunctional protein known for its role as substrate-binding subunit of an E3 ubiquitin-ligase that, among others, targets the α-subunits of the transcription factors hypoxia-inducible factors (HIFs-α) for proteasomal degradation. Biallelic inactivation of the *VHL* gene would thereof result in HIF stabilization with subsequent up-regulation of target genes involved in angiogenesis, proliferation, apoptosis and metabolism. The *VHL* gene alteration predisposes to several highly vascularized benign and malignant tumors, including retinal and central nervous system hemangioblastomas, pheochromocytomas and clear-cell renal cell carcinomas (ccRCCs) (Barontini and Dahia 2010; Barrisford et al. 2011; Bader and Hsu 2012). In the last decade, many HIF-independent activities have been also described for the VHL protein in many key cellular processes, such as regulation of extracellular matrix, senescence, apoptosis, RNA stability, endocytosis and gene transcription (Hsu 2012; Richard et al 2013). Among the VHL-associated tumors, ccRCC is the major cause of death related to this disease (Maher et al. 2011). Those VHL-associated renal carcinomas have histology of the clear cell type and preferentially tend to be multifocal, bilateral and cystic (Barontini and Dahia 2010; López 2013). Around two-thirds of the patients with *VHL* germ-line mutations develop early age ccRCC and are at risk of renal cancer development of approximately 70% (Lonser et al 2003). Currently, abdominal ultrasound, computerized tomography and magnetic resonance imaging allow the monitoring of the patients in order to diagnose ccRCC at the earliest stage (Maher et al 2011). Nevertheless, the pathophysiological mechanism on the basis of the correlation between kidney cancer and VHLD is still unknown. The discovery of a new non-invasive biomarker could be helpful not only to support currently used techniques for the clinical monitoring of VHLD patients, but also to obtain a better knowledge of the molecular basis of ccRCC onset in VHLD.

Since ccRCC usually arises from the epithelial cells of the proximal part of the renal tubule lining the urinary tract that contributes to urine formation and given that urine samples can be easily and copiously available in a non-invasive manner, we decided to focus on the urinary proteome (Bodmer et al. 2002). In order to identify new non-invasive biomarkers, we compared the proteome of urine samples provided by healthy volunteers, VHLD-affected subjects and patients with ccRCC. Interestingly we identified proteins characteristics of VHLD or sporadic ccRCC, able to differentiate them from healthy subjects. In particular, among all the differentially expressed proteins, we focus our attention on two proteins that could discriminate between VHLD patients with o without ccRCC history, namely A1AT (alpha-1-antitrypsin) and APOH (beta-2-glycoprotein-1) that could therefore represent promising markers for renal carcinogenesis in VHLD patients.

Materials and methods

Population of the Study

From October 2010 to May 2012 patients with VHLD (with or without ccRCC history) and with sporadic ccRCC were enrolled in this study and provided urine specimens. Concomitantly a control group composed by 6 male and 2 female healthy volunteers with a mean age of 40.5 years provided urine specimens. The protocol was approved by the local research ethical committee and done according to Helsinki Declaration principles. All participants in the protocol signed a declaration of informed consent. A total of 4 male and 5 female patients with VHLD with a mean SD age of 36.4 years, 3 male and 6 female patients with ccRCC with a mean SD age of 69.4 years were enrolled. Among the 9 VHL patients, 4 experienced at minimum one ccRCC before urine collection (**Table 1**). Samples were collected in sterile tubes and processed within 2 hours of sampling.

Chemicals and reagents

Unless otherwise stated, all materials were from Sigma-Aldrich (Milan, Italy). DC Protein assay kit, acrylamide, agarose, ready-made immobilized pH gradient (IPG) strip (7- cm IPG strips, pH 3-10NL) from Bio-Rad (Hercules, CA, USA). Ampholine pH 3.5–10 and horseradish-peroxidase labeled antibodies against rabbit or mouse IgG were obtained from GE Healthcare (Milan, Italy). The slide-A-lyzer mini dialysis unit plus microtube was purchased from Pierce (Rockford, IL, USA). Antibody against APOH was purchased from Abcam (Cambridge, UK).

Urine Processing

Urine samples were centrifuged at 700 x g for 20 minutes at 10°C to remove exfoliated urothelial cells and debris. Proteins from supernatant were precipitated by acetone 1:1. Pellets were solubilized in lysis buffer (urea 9M, CHAPS 4%, Na_3VO_4 1mM, DTT 80mM, protease inhibitors and nuclease). Samples were incubated overnight at 4°C and spun down at 13,800 g for 10 min at 4°C. The clear supernatant was removed, quantified with DC Protein assay kit and stored at -20°C until analysis.

Two-dimensional gel electrophoresis (2DE)

200 µg of proteins for each sample were dialyzed against fresh lysis buffer with slide-A-lyzer mini dialysis unit plus microtube according to manufacturer's instructions. 2DE was performed using ready-made IPG strip (7-cm IPG strips, pH 3-10NL) and gel stained with Colloidal Coomassie as already described (Mandili G et al. 2011).

Image analysis

Image analysis for 2DE experiments was performed using PD-Quest software (version 7.2, Bio-

Rad) according to the manufacturer's instructions, as previously described (Mandili G et al. 2011).

Protein identification by mass spectrometry and database search

Coomassie G-stained spots were excised from 2DE gels; destaining, in-gel enzymatic digestion and MALDI-TOF analysis were performed as previously described (Mandili G et al. 2011). The following parameters were used in the searches using the free program MASCOT (www.matrixscience.com): database SWISSPROT, taxa Homo sapiens, trypsin digest, one missed cleavage by trypsin, carbamidomethylation of cysteine as fixed modification, methionine oxidation as variable modification and maximum error allowed 100 ppm. Only proteins with a Mascot score greater than 56 were considered for further analyses.

Western blotting

Lysates containing equal amounts of proteins (10 µg), added with Laemmli buffer (Tris-HCl 60mM pH 6.8, SDS 2% w/v, glycerol 10% v/v), were subjected to SDS-PAGE (10% gel). The separated proteins were transferred to a nitrocellulose membrane. The blot was probed using antibody against APOH or A1AT. Immunoreactivity was detected using an enhanced chemiluminescence kit. Densitometric analysis of the bands was performed using free ImageJ software (http://imagej.net).

Statistical analysis

Data from image analysis were used as values of protein expression. Two-sided Student's t test was used to evaluate the significance of the variations of protein expression. Statistical significance was set at p value ≤ 0.05. For 2DE experiments proteins were classified as differentially expressed if ratio in spot intensity was greater than 1.5-fold (protein over-represented) or lower than 0.5- fold (protein under-represented).

The ROC (Receiver Operating Characteristic) curves were computed using GraphPad Prism 6 Software.

Results

Comparative analysis of 2D gels of urine samples from VHLD patients, healthy subjects and ccRCC patients

To obtain a better insight into renal carcinogenesis in patients with VHLD and identify new potential markers for this disease, we investigated the differences in protein profiles between urine samples from healthy subjects, VHLD and ccRCC patients. All paired conditions were considered. All the differentially expressed proteins identified are listed in **Tables 2** and **3** and indicated in **Figure 1**.

Comparative analysis of 2D gels among VHLD urine samples

In the comparison between the 2D maps obtained from urine samples of the two VHLD conditions (positive or negative history of ccRCC), image analysis revealed 3 spots differentially expressed corresponding to 3 unique proteins: AMY1 (alpha-amylase 1/2B/P), AMYP/2B (pancreatic alpha-amylase/alpha-amylase 2B) and APOH (beta-2-glycoprotein-1) (**Table 3**).

Comparative analysis of 2D gels of VHLD patients and healthy subjects urine samples

The comparison of 2D maps of urinary proteins from VHLD patients and healthy volunteers revealed 7 spots significantly modulated (**Table 2**) corresponding to ALBU (serum Albumin, 6 spots) and KNG1 (kininogen1).

The comparison between the two VHLD subgroups and healthy subjects was also performed. Interestingly only 2 spots, both identified as ALBU, were found to be differentially expressed between VHLD patients who didn't experience ccRCC and healthy subjects (**Table 3**). Indeed, 8 spots were differentially expressed between VHLD patients who experienced ccRCC and healthy subjects: ALBU (3 spots), A1AT (alpha-1-antitrypsin), IGKC (Ig kappa chain C), ITIH4 (inter-alpha-trypsin inhibitor heavy chain 4, 2 spots) and SEN54 (tRNA splicing endonuclease subunit Sen54) (**Table 3**).

All comparisons are visualized in **Figure 2**.

Comparative analysis of 2D gels of urine samples from VHLD and ccRCC patients

In the comparison of 2D maps of urinary proteins from VHLD and ccRCC patients, 17 spots were differentially expressed (**Table 2**) and were referred to 10 proteins: ALBU (3 spots), IGKC (2 spots), IN80C (INO80 complex subunit C), KCA10 (potassium voltage-gated channel subfamily A member 10), SDCB1 (syntenin-1), IGHG1 (3 spots), TRFE (transferrin, 2 spots), AMBP (protein AMBP, 2 spots), LIPR3 (pancreatic lipase-related protein 3) and CATD (cathepsin D). The comparison between the two VHLD subgroups and the sporadic ccRCC patients was also performed. Unexpectedly the urine proteome of VHLD patients who developed renal cancer showed a higher number of differences in protein profile than those without renal cancer history when compared with the urine of sporadic ccRCC patients: 15 spots corresponding to IGKC, IN80C, SDCB1, IGHG1, ITIH4, LAC1/2/3 (Ig lambda-1/2/3 chain C regions), AMBP, SEN54, CATD (**Table 3**). Four of those proteins were also found to be differentially expressed comparing the urine of VHLD patients who had not developed cancer with the urine from sporadic ccRCC patients: IN80C, IGHG1, LAC1/2/3, AMBP (derived from 5 spots, **Table 3**).

All comparisons are visualized in **Figure 2**.

Comparative analysis of 2D gels of urine samples from ccRCC patients and healthy subjects

Eleven spots displayed differential levels in the urine of patients with ccRCC if compared to the urine of healthy subjects. They referred to 6 proteins (**Table 2**): IGHG1 (5spots), IN80C, KCA10, SDCB1, AMBP (2 spots) and CATD. All comparisons are visualized in **Figure 2**.

APOH and A1AT validation

Among all the differentially expressed proteins, A1AT and APOH were chosen for western blotting validation for their known association with tumors.

The western blot in **Figure 3a** and the histogram in **Figure 3b** showed the increase of A1AT levels in the urine of VHLD patients who had developed renal cancer in comparison to those who did not (p= 0.0003) and also to patients with ccRCC (p= 0.0088).

A similar result was observed analyzing APOH levels. As shown in **Figure 3a** and in the histogram in **Figure 3c**, APOH levels were considerably higher in the urine of patients who had developed renal cancer if compared with the levels in the urine of VHLD patients who had never developed cancer (p= 0.012), healthy subjects (p= 0.018) and patients with sporadic ccRCC (p= 0.0005).

Notably the statistical comparative analysis of the A1AT levels between the two VHLD subgroups, performed by the ROC curve analysis (cut-off value=0.34, p=0.014), revealed we were able to identify all true negative (5 out of 5 VHL patients with no ccRCC) and all true positive values (4 out of 4 VHL patients that had developed ccRCC). The same analysis was performed for APOH levels (cut-off value=1.79, p = 0.050); it allowed to identify 3 (out of 4) VHLD patients that had developed ccRCC, correctly discriminating those patients from the true negative patients (5 out of 5 detected).

Discussion

Clinical diagnosis routinely relies on urine samples since the collection process is easy and non-invasive (Gebregiworgis et al. 2016). Since urine is secreted by the kidney through a finely controlled urination process, changes in urinary proteome can be generally considered an indication of kidney injury (Bodmer et al. 2002).

The VHLD is a rare neoplastic syndrome caused by germline mutations of the suppressor gene *VHL*. Despite a wide range of pathological outcomes, ccRCC is the most frequent cause of morbidity and mortality among the patients affected by VHLD (López 2013).

In order to identify a new useful marker to assess ccRCC risk in VHLD patients and to investigate the mechanism of renal carcinogenesis, we performed a proteomic analysis comparing urine samples provided by VHLD patients with or without a history of ccRCC, sporadic ccRCC patients and healthy subjects.

The comparison among VHLD patients, ccRCC patients and healthy subjects revealed changes in proteins involved in immune system (IGHG1, IGKC, AMBP), cell motility and proliferation (CATD), cell permeability (KNG1 and KCA10), lipid metabolism (LIPR3), signal transduction (SDCB1), chromatin remodeling (INO80) and ion transport (ALBU, TRFE).

Among all the identified proteins, alpha-1-antitrypsin (A1AT) and beta-2-glycoprotein 1 (APOH) were strongly over-abundant in the urine of VHLD patients with history of ccRCC in comparison to the urine of all the other conditions.

A1AT is a protease inhibitor that protects tissues from the inflammatory cells proteases. A huge increase in A1AT urinary excretion was described in a model of acute kidney injury (Zager et al. 2014) and in patients affected by renovascular hypertension, probably because of local changes in the kidney (Lisowska-Myjak 2005). Furthermore, high A1AT levels have also been described in association with tumors (bladder cancer (Miyake et al. 2013), colorectal cancer (Pérez-Holanda et al. 2014), cervical cancer (Boichenko et al. 2014) and lung cancer (Liang et al. 2015)), suggesting its involvement in tumor development even though its role has not yet been understood. We can hypothesize that local structural changes occurring in the kidney following VHLD-induced ccRCC could cause increased excretion of A1AT in the urine of VHLD patients with ccRCC history, leading to the high level observed in the present study. Impressively, increased A1AT levels were also described in another lesser common benign lesion that may occur as a manifestation of VHLD, the papillary cystadenoma of the epididymis (Gilcrease et al. 1995), suggesting a direct link between A1AT overexpression and *VHL* mutation.

APOH is a multifunctional protein belonging to the apolipoprotein family. It mainly prevents the activation of the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells. A role for APOH in the modulation of angiogenesis has also been described (Passam et al. 2010) and this could explain its relationship with the onset of ccRCC, a highly vascularizated tumor, and VHLD. According to our results, APOH was previously identified as one of the major urinary proteins over-excreted by patients with several renal tubular diseases (Norden et al. 1991). Moreover, APOH levels were already reported to reflect tumor progression in several cancers, including acute myeloid leukemia (Lee et al. 2012), breast cancer (Chung et al. 2014), non-small lung cancer (Pietrowska et al. 2014) and hepatocellular carcinoma (Jing et al 2010).

Interestingly we also identified 6 proteins which differentiated urine of sporadic ccRCC patients from those of healthy subjects. The discovery of new ccRCC biomarkers is still an open field and although ultrasonography represents a major tool for diagnosis and screening of renal masses, not invasive biomarkers could became helpful complementary research methods in diagnosis and prognosis (Giribaldi et al. 2013). Obviously validation of these proteins as possible markers will be necessary, but our observations could represent the bases of future studies.

In conclusion, our results suggest A1AT and APOH as promising markers for ccRCC in VHLD patients. A1AT and APOH levels in urine might contribute to identify patients who are more susceptible to develop cancer, allowing a better follow-up of VHLD patients.

Because of the extremely rarity of this pathology, our work, even with only 9 VHLD patients, could represent an important contribution in the VHLD study, even though further analysis on a larger cohort of patients will be necessary to fully validate our observations and deeply elucidate the role of A1AT and APOH in carcinogenesis. With a future perspective study will be possible the evaluation of diagnostic and prognostic power of this two markers and their correlation with factors such as sex, age, smoking, BMI, hypertension and for cases, tumor grade, stage and size.

Moreover our discovery of the involvement of A1AT and APOH in the carcinogenesis of ccRCC opens new scenarios for the study of this tumor even in the perspective of the development of new targeted therapies.

Geolocalization data

Italy, Piemonte, Turin. Latitude 45.05. Longitude 7.6667

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Abbreviations

VHL (Von Hippel-Lindau), VHLD (Von Hippel-Lindau disease), HIF (hypoxia-inducible factor), ccRCC (clear-cell renal cell carcinoma), A1AT (alpha-1-antitrypsin), APOH (beta-2-glycoprotein 1), 2DE (bidimensional electrophoresis)

Conflict of interest

The authors declare that they have no conflict of interest

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Table 1. Patients characteristics

Pts	date of birth	gender	date of urine collection	familiarity	RCC	others VHL-related pathologies	mutation
VHL1	22/06/78	F	01/12/2011	no	no	central nervous system and retinal hemangioblastomas, bilateral pheochromocytomas, pancreatic cysts sx	R161Q (CGA-CAA) exon 3
VHL2	19/06/67	F	01/12/2011	yes (mother)	pT1a Gx	central nervous system and retinal hemangioblastomas, pancreatic cysts	_
VHL3	21/10/87	M	07/12/2011	no	no (october 2014: pT1aG1)	central nervous system and retinal hemangioblastomas, bilateral pheochromocytomas, multiple pancreatic cysts	499C>T (p.Arg167Trp) phenotype 2B
VHL5	19/09/84	M	08/05/2012	yes (father, brother)	pT1aGx	pancreatic cysts, retinal angioma	_
VHL6	24/11/73	M	08/05/2012	yes (father, brother)	pT1aGx	central nervous system and retinal hemangioblastomas, pancreatic and renal cysts	_
VHL8	05/05/83	F	08/05/2012	no	no (january 2014: pT1aGx)	central nervous system and retinal hemangioblastomas, pancreatic and renal cysts	N78S
VHL9	16/11/88	F	08/05/2012	yes (father)	no	retinal hemangioblastomas, pancreatic and renal cysts, endolymphatic sac tumors	Deletion exon 1
VHL10	24/09/84	M	08/05/2012	yes (mother, brother)	no	bilateral pheochromocytomas	heterozigous missense 467A>C (p. Y156S) in exon 3
VHL11	04/08/54	F	08/05/2012	yes (two sons)	pT1aG2	retinal hemangioblastomas, bilateral pheochromocytomas, pancreatic neuroendocrine tumours	heterozigous missense 467A>C (p. Y156S) in exon 3
R1	17/05/55	F	18/10/2010	_	pT2bG3	_	_
R4	27/02/67	F	28/10/2010	_	pT3aG2	_	_
R5	28/01/35	F	03/11/2010	_	pT1bNo	_	_
R9	27/04/62	M	28/04/2011	_	pT2aG3	_	_
R11	26/09/32	F	05/07/2011	_	pT3aG2	_	_
R12	31/08/37	M	23/11/2011	_	pT3aG4	_	_
R13	04/04/52	M	21/12/2011	_	pT3aG2N0	_	_
R17	15/02/37	F	25/01/2012	_	pT3aNx	_	_
R21	19/093/1933	F	12/03/2012	_	pT2aG2	_	_

Table 2. Identified proteins in VHLD patients, ccRCC patients and healthy subjects' urine

Acronym (ID), Swissprot accession number, name, molecular weight (MW), isoelectric point (pI), Spot number according to spot position in 2DE in Figure1, Mascot score, sequence coverage (percentage of identified sequence of the matched protein) and number of matched peptides out of 25 are indicated

Proteins differentially expressed in urine of VHLD patients *vs* healthy subjects								
ID	**Accession Number**	**Name**	**MW**	**pI**	**Nr spot**	**Mascot Score**	**Coverage %**	**Matched peptides**
ALBU	P02768	Serum albumin	71317	5,92	606	222	36	19
					705	175	24	14
					2603	245	33	21
					3704	292	39	22
					3803	243	38	20
					4804	254	36	20
KNG1	P01042	Kininogen-1	72996	6,34	705	175	22	11
Proteins differentially expressed in urine of VHLD patients *vs* ccRCC patients								
ID	**Accession Number**	**Name**	**MW**	**pI**	**Nr spot**	**Mascot Score**	**Coverage %**	**Matched peptides**
ALBU	P02768	Serum albumin	71317	5,92	2603	245	33	21
					4702	124	23	11
					4804	254	36	20
IGKC	P01834	Ig kappa chain C region	11773	5,58	3301	68	66	4
					8201	68	66	4
IN80C	Q6PI98	INO80 complex subunit C	20630	10,03	3401	57	30	4
KCA10	Q16322	Potassium voltage-gated channel subfamily A member 10	58147	4,85	3601	61	18	6
SDCB1	O00560	Syntenin-1	32595	7,05	9501	74	31	5
IGHG1	P01857	Ig gamma-1 chain C region	36596	8,46	9501	74	25	5
					9704	84	36	7
					9705	77	35	7
TRFE	P02787	Serotransferrin	79294	6,81	9912	209	28	19
					9913	183	29	18
AMBP	P02760	Protein AMBP	39886	5,95	9932	61	23	7

					9951	59	22	6
LIPR3	Q17RR3	Pancreatic lipase-related protein 3			9952	57		
CATD	P07339	Cathepsin D	45037	6,1	9958	84	25	8
Proteins differentially expressed in urine of ccRCC patients *vs* healthy subjects								
ID	**Accession Number**	**Name**	**MW**	**pI**	**Nr spot**	**Mascot Score**	**Coverage %**	**Matched peptides**
IN80C	Q6PI98	INO80 complex subunit C	20630	10,03	3401	57	30	4
KCA10	Q16322	Potassium voltage-gated channel subfamily A member 10	58147	4,85	3601	61	18	6
SDCB1	O00560	Syntenin-1	32595	7,05	9501	74	31	5
IGHG1	P01857	Ig gamma-1 chain C region	36596	8,46	9501 9704 9705 9706 9935	74 84 77 93 82	25 36 35 36 36	5 7 7 7 8
AMBP	P02760	Protein AMBP	39886	5,95	9932 9951	61 59	23 22	7 6
CATD	P07339	Cathepsin D	45037	6,1	9958	84	25	8

Table 3. Identified proteins in VHLD patients with or without ccRCC history, ccRCC patients and healthy subjects' urine

Acronym (ID), Swissprot accession number, name, molecular weight (MW), isoelectric point (pI), Spot number according to spot position in 2DE in Figure1, Mascot score, sequence coverage (percentage of identified sequence of the matched protein) and number of matched peptides out of 25 are indicated

Proteins differentially expressed in urine of VHLD patients with ccRCC history *vs* healthy subjects								
ID	**Accession Number**	**Name**	**MW**	**pI**	**Nr spot**	**Mascot Score**	**Coverage %**	**Matched peptides**
ALBU	P02768	Serum albumin	71317	5,92	606 2603 3704	222 245 292	36 33 39	19 21 22
A1AT	P01009	Alpha-1-antitrypsin	46878	5,37	707	129	33	11
IGKC	P01834	Ig kappa chain C	11773	5,58	9929	63	66	4
ITIH4	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	103521	6,51	9919 9921	78 60	17 13	10 6
SEN54	Q7Z6J9	tRNA-splicing endonuclease subunit Sen54	59296	8,03	9946	57	12	6
Proteins differentially expressed in urine of VHLD patients without ccRCC history *vs* healthy subjects								
ID	**Accession Number**	**Name**	**MW**	**pI**	**Nr spot**	**Mascot Score**	**Coverage %**	**Matched peptides**
ALBU	P02768	Serum albumin	71317	5,92	606 2603	222 245	36 33	19 21
Proteins differentially expressed in urine of VHLD patients with ccRCC history *vs* VHLD patients without ccRCC history								
ID	**Accession Number**	**Name**	**MW**	**pI**	**Nr spot**	**Mascot Score**	**Coverage %**	**Matched peptides**
AMY1	P04745	Alpha-amylase 1/2B/P	58415	6.47	8701	179	33	12
AMYP/2B	P04746/ P19961	Pancreatic alpha-amylase/ Alpha-amylase 2B	58354/ 58300	6,60/ 6,64	8702	57	17	7
APOH	P02749	Beta-2-glycoprotein 1	39584	8.34	8701	179	33	7
Proteins differentially expressed in urine of VHLD patients with ccRCC history *vs* ccRCC patients								
ID	**Accession Number**	**Name**	**MW**	**pI**	**Nr spot**	**Mascot Score**	**Coverage %**	**Matched peptides**
IGKC	P01834	Ig kappa chain C region	11773	5,58	3301 9929	68 63	66 66	4 4

IN80C	Q6PI98	INO80 complex subunit C	20630	10,03	3401	57	30	4
SDCB1	O00560	Syntenin-1	32595	7,05	9501	74	31	5
IGHG1	P01857	Ig gamma-1 chain C region	36596	8,46	9501 9704 9935	74 84 82	25 36 36	5 7 8
ITIH4	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	103521	6,51	9919 9921	78 60	17 13	10 6
LAC1/2/3	P0CG04/5/6	Ig lambda-1/2/3 chain C regions	11512/ 11458/ 11402	7,89/ 6,92/ 6,92	9931	63	65	4
AMBP	P02760	Protein AMBP	39886	5,95	9932 9951	61 59	23 22	7 6
SEN54	Q7Z6J9	tRNA-splicing endonuclease subunit Sen54	59296	8,03	9946	57	12	6
CATD	P07339	Cathepsin D	45037	6,1	9958	84	25	8

Proteins differentially expressed in urine of VLHD patients without ccRCC history *vs* ccRCC patients

ID	Accession Number	Name	MW	pI	Nr spot	Mascot Score	Coverage %	Matched peptides
IN80C	Q6PI98	INO80 complex subunit C	20630	10,03	3401	57	30	4
IGHG1	P01857	Ig gamma-1 chain C region	36596	8,46	9705	77	35	7
LAC1/2/3	P0CG04/5/6	Ig lambda-1/2/3 chain C regions	11512/ 11458/ 11402	7,89/ 6,92/ 6,92	9931	63	65	4
AMBP	P02760	Protein AMBP	39886	5,95	9932 9951	61 59	23 22	7 6

Legends to figures

Figure 1. Representative 2DE map of urinary proteins

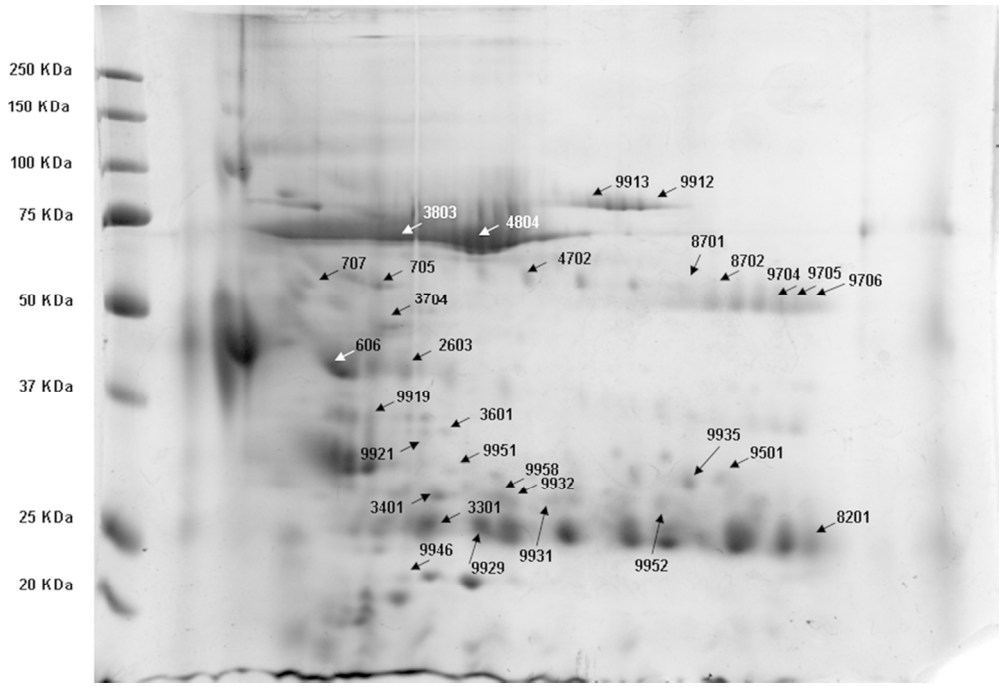
The spots corresponding to all the identified proteins differentially expressed among the analyzed clinical conditions are indicated on the figure by arrows and listed in Tables 2 and 3.

Figure 2. Modulated proteins in VHLD in comparison to other conditions

a) Venn diagram highlighting proteins modulated in the comparison among VHLD patients, ccRCC patients and healthy subjects. b) Venn diagram highlighting proteins modulated in the comparison among VHLD patients with (VHL K) or without (VHL no K) ccRCC history, ccRCC patients and healthy subjects

Figure 3. A1AT and APOH validation

a) Representative western blotting images of A1AT and APOH in urine provided by healthy volunteers (C1 and C2), VHLD patients without ccRCC history (VHL no k, V1), VHLD patients with ccRCC history (VHL k, V2) and patients with sporadic renal cancer (R1 and R2). b) and c) show the corresponding distribution of the data obtained from the densitometric analysis of A1AT and APOH expression respectively (* $p<0.05$; ** $p<0.01$; *** $p<0.005$).



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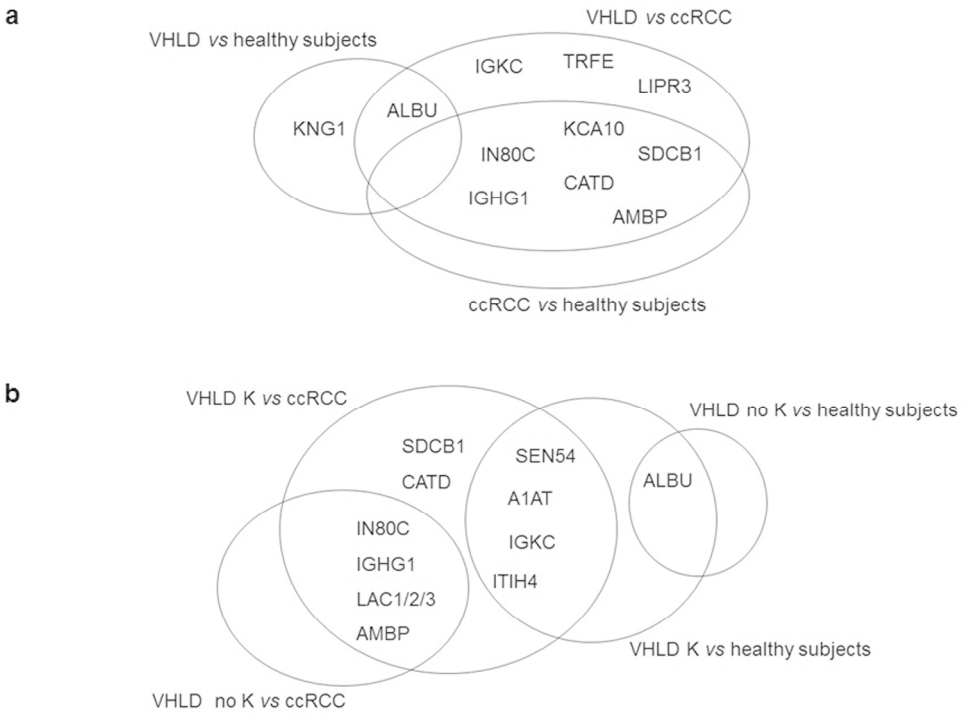

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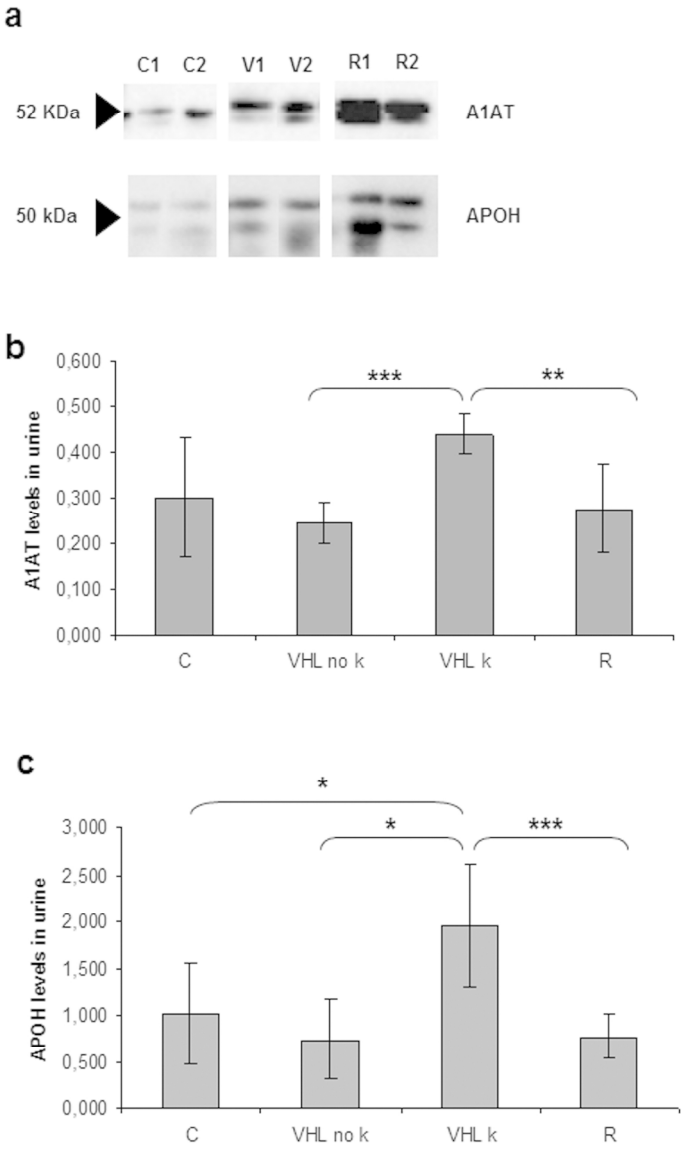

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